



THE UNIVERSITY *of* EDINBURGH

## Edinburgh Research Explorer

# Preferential Expression of Integrin v8 Promotes Generation of Regulatory T Cells by Mouse CD103<sup>+</sup> Dendritic Cells

### Citation for published version:

Païdassi, H, Acharya, M, Zhang, A, Mukhopadhyay, S, Kwon, M, Chow, C, Stuart, LM, Savill, J & Lacy-Hulbert, A 2011, 'Preferential Expression of Integrin v8 Promotes Generation of Regulatory T Cells by Mouse CD103<sup>+</sup> Dendritic Cells', *Gastroenterology*, vol. 141, no. 5, pp. 1813-1820.  
<https://doi.org/10.1053/j.gastro.2011.06.076>

### Digital Object Identifier (DOI):

[10.1053/j.gastro.2011.06.076](https://doi.org/10.1053/j.gastro.2011.06.076)

### Link:

[Link to publication record in Edinburgh Research Explorer](#)

### Document Version:

Peer reviewed version

### Published In:

Gastroenterology

### Publisher Rights Statement:

Published in final edited form as:  
Gastroenterology. 2011 November; 141(5): 1813–1820.  
Published online 2011 July 13. doi: 10.1053/j.gastro.2011.06.076

### General rights

Copyright for the publications made accessible via the Edinburgh Research Explorer is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

### Take down policy

The University of Edinburgh has made every reasonable effort to ensure that Edinburgh Research Explorer content complies with UK legislation. If you believe that the public display of this file breaches copyright please contact [openaccess@ed.ac.uk](mailto:openaccess@ed.ac.uk) providing details, and we will remove access to the work immediately and investigate your claim.



Published in final edited form as:

*Gastroenterology*. 2011 November ; 141(5): 1813–1820. doi:10.1053/j.gastro.2011.06.076.

## Preferential Expression of Integrin $\alpha\beta 8$ Promotes Generation of Regulatory T Cells by Mouse CD103<sup>+</sup> Dendritic Cells

Helena PAÏDASSI<sup>1,2</sup>, Mridu ACHARYA<sup>1</sup>, Ailiang ZHANG<sup>2</sup>, Subhankar MUKHOPADHYAY<sup>1,3</sup>, Manjue KWON<sup>1</sup>, Camille CHOW<sup>1</sup>, Lynda M. STUART<sup>1</sup>, John SAVILL<sup>2</sup>, and Adam LACY-HULBERT<sup>1</sup>

<sup>1</sup>Program of Developmental Immunology, Department of Pediatrics, Massachusetts General Hospital/Harvard Medical School, Boston, MA, USA

<sup>2</sup>Queen's Medical Research Institute/MRC Centre for Inflammation Research, University of Edinburgh, Edinburgh, UK

<sup>3</sup>Sir William Dunn School of Pathology, University of Oxford, Oxford, UK

### Abstract

**Background & Aims**—Immune responses in the intestine are controlled by regulatory T cells (Treg cells), which prevent inflammation in response to commensal bacteria. A specific population of intestinal dendritic cells (DCs), marked by expression of CD103, generate Tregs more efficiently than other DC populations, through mechanisms that involve retinoic acid and transforming growth factor (TGF)- $\beta$ . However, it is not clear how CD103<sup>+</sup> DCs are specialized for this function. We investigated the ability of CD103<sup>+</sup> DCs to promote Treg generation through activation of TGF- $\beta$  and the role of integrins with the  $\alpha$  subunit in this process.

**Methods**—Naïve T cells were cultured with purified DCs from mesenteric lymph nodes (MLNs) or intestines of wild-type and  $\alpha$  conditional knockout mice, to assess generation of Treg cells. Antigens were administered orally to mice and antigen-specific generation of Treg cells was measured in intestinal tissues. Expression of the integrin  $\alpha$  subunit was measured in purified subpopulations of DCs by quantitative PCR and immunoblot analyses.

**Results**—In vitro, CD103<sup>+</sup> DCs generated more Treg cells in the presence of latent TGF- $\beta$  than other MLN DCs. Efficient generation of Treg cells required expression of the integrin  $\alpha$  subunit by DCs; mice that lacked  $\alpha$  in immune cells did not convert naïve T cells to intestinal Treg cells in response to oral antigen. CD103<sup>+</sup> DCs derived from the MLNs selectively expressed high levels of integrin  $\alpha\beta 8$ , compared with other populations of DCs.

**Conclusions**—Expression of  $\alpha\beta 8$  is required for CD103<sup>+</sup> DCs to become specialized and activate latent TGF- $\beta$  and generate Treg cells during the induction of tolerance to intestinal antigens in mice.

© 2011 The American Gastroenterological Association. Published by Elsevier Inc. All rights reserved.

Correspondence to: Dr Adam Lacy-Hulbert, Program of Developmental Immunology, Massachusetts General Hospital GRJ1402, 55 Fruit Street, Boston 02114, Tel: 617 643 5346, Fax: 617 724 3248, alacy-hulbert@partners.org.

**Disclosures:** The authors declare they have no conflicting interests.

**Author Contributions:** AL-H, JS and HP designed the study, interpreted the data and wrote the manuscript and AL-H and JS contributed equally as senior authors. HP, MA, AZ, SM, MK and CC performed experiments and acquired data. LMS helped to design the study, interpret data and write the manuscript.

**Publisher's Disclaimer:** This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

## Keywords

FoxP3; signaling; microbiota; immune regulation

## Introduction

In the gastrointestinal tract, the immune system must protect against potential pathogens whilst preventing destructive inflammatory responses to the host's commensal microbiota<sup>1</sup>. This is achieved in part through a complex and active network of specialized immune cells and regulatory cytokines<sup>2</sup>. Breakdown of this dynamic balance between immune response and regulation is considered a major contributor to Inflammatory Bowel Disease (IBD)<sup>3</sup>.

Regulatory T cells (Tregs), characterized by expression of the forkhead transcription factor FoxP3, are an important component of intestinal immune regulation and loss of Tregs causes spontaneous colitis, driven by microbial products in the gut<sup>4</sup>. A major population of FoxP3<sup>+</sup> Tregs develop from self-reactive cells in the thymus and these 'naturally occurring' nTregs appear to regulate autoimmunity<sup>5</sup>. FoxP3<sup>+</sup> expression can also be stimulated in naïve T cells in the periphery, providing an additional source of Tregs<sup>6, 7</sup> with a different potential antigen repertoire to nTregs. Development of these 'adaptive' or induced Tregs (iTregs) requires low levels of antigen and costimulation, combined with the immunoregulatory cytokine TGF- $\beta$ <sup>8, 9</sup>. The generation of iTregs may be of particular importance in mucosal tissues such as the intestine, as it provides a mechanism by which tolerance can be induced against dietary antigens and the commensal microflora<sup>4</sup>.

Recently, a specific population of dendritic cells (DCs) from the mouse intestinal lamina propria and MLNs, marked by surface expression of  $\alpha$ E integrin chain (CD103), were implicated in the generation of iTregs<sup>10, 11</sup>. In co-culture with naïve CD4<sup>+</sup> T cells, CD103<sup>+</sup> DCs produced more FoxP3<sup>+</sup> Tregs than their CD103<sup>-</sup> counterparts<sup>10, 11</sup>. The induction of Tregs by these DCs could be prevented by neutralization of TGF- $\beta$  and retinoic acid (RA); conversely addition of exogenous TGF- $\beta$  and/or RA promoted Treg generation to similar levels in both CD103<sup>+</sup> and CD103<sup>-</sup> DCs. Therefore the preferential induction of FoxP3<sup>+</sup> Tregs by CD103<sup>+</sup> DCs was due to promotion of TGF- $\beta$  and RA signaling to T cells. It has been shown that CD103<sup>+</sup> DCs produce high levels of RA which in turn stabilizes TGF- $\beta$ -induced FoxP3 expression in T cells whilst inhibiting Th17 differentiation. However, little is known of the contribution of CD103<sup>+</sup> DCs to TGF- $\beta$  production, despite the requirement for TGF- $\beta$  for the effects of RA. We therefore set out to determine whether CD103<sup>+</sup> DCs were also specialized for promoting TGF- $\beta$  signaling.

Here we present evidence that enhanced generation of FoxP3<sup>+</sup> Tregs by CD103<sup>+</sup> DCs is also due to their ability to activate latent TGF- $\beta$ . Thus, MLN CD103<sup>+</sup> DCs generate higher numbers of Tregs than CD103<sup>-</sup> DCs when cultured in the presence of latent, but not active TGF- $\beta$ . This is dependent on DC expression of  $\alpha$ v integrins *in vitro* and *in vivo*, and mice lacking  $\alpha$ v in the immune system are unable to generate intestinal Tregs from naïve T cells in response to oral antigen. Furthermore, MLN CD103<sup>+</sup> DCs alone express the  $\alpha$ v $\beta$ 8 integrin heterodimer. Hence these data show that the expression of  $\alpha$ v integrins by intestinal DCs is critical for the generation of FoxP3<sup>+</sup> Tregs in response to intestinal antigens, and that the CD103<sup>+</sup> subset of DCs are uniquely specialized for this function through the expression of  $\alpha$ v $\beta$ 8 allowing activation of latent TGF- $\beta$ .

## Results and Discussion

### CD103<sup>+</sup> MLN DCs activate latent TGF- $\beta$ to generate FoxP3<sup>+</sup> Tregs

In agreement with previous studies<sup>10, 11</sup>, we found that CD103<sup>+</sup> DCs from mouse MLNs generated on average twice as many FoxP3<sup>+</sup> T cells in *in vitro* co-culture than their CD103<sup>-</sup> counterparts (Figure 1A). This was dependent on TGF- $\beta$  as TGF- $\beta$  blocking antibodies completely prevented Treg generation by both CD103<sup>+</sup> and CD103<sup>-</sup> DCs (Figure 1A). FoxP3 induction was also significantly impaired when DCs and T cells were cultured in serum free medium (Figure 1B–C), despite comparable T cell proliferation to that seen in serum-replete medium (data not shown), indicating that the majority of TGF- $\beta$  responsible for Treg generation was derived from serum in the culture medium, rather than endogenous production by DCs or T cells. However, it is worth noting that even in serum-free conditions, CD103<sup>+</sup> DCs from control mice produced more Tregs than CD103<sup>-</sup> DCs (Figure 1C).

Based on these data, we reasoned that CD103<sup>+</sup> DCs may be better able to utilize exogenous TGF- $\beta$  to promote FoxP3 expression in T cells. TGF- $\beta$  is synthesized as an inactive ‘latent’ precursor which must be dissociated from binding proteins before it can engage TGF- $\beta$  receptors, a process known as TGF- $\beta$  activation. When MLN DCs were cultured with purified latent TGF- $\beta$ , CD103<sup>+</sup> DCs again induced significantly more FoxP3<sup>+</sup> Tregs than CD103<sup>-</sup> cells (Figure 1B–C). These data suggested to us that the difference in Treg generation between CD103<sup>+</sup> and CD103<sup>-</sup> DCs might be explained in part by a differential ability to activate latent TGF- $\beta$ .

The preferential generation of Tregs by CD103<sup>+</sup> Dcs has previously been linked to expression of high levels of *aldh1a2* which confers on this DC subset the ability to synthesize and secrete RA, which promotes Treg generation<sup>10, 11</sup>. Consistent with these studies, CD103<sup>+</sup> and CD103<sup>-</sup> DCs produced equivalent proportions of FoxP3<sup>+</sup> Tregs when cultured with active TGF- $\beta$  and RA (Figure 1D). However, addition of RA was not sufficient to allow CD103<sup>-</sup> DCs to efficiently generate Tregs in response to latent TGF- $\beta$ . Therefore, the increased induction of FoxP3<sup>+</sup> Tregs by CD103<sup>+</sup> DCs was in part due to increased activation of latent TGF- $\beta$ , independent of their ability to produce RA.

### Intestinal CD103<sup>+</sup> DCs activate TGF- $\beta$ via $\alpha$ v integrins

$\alpha$ v integrins are important physiological activators of latent TGF- $\beta$  and mice deficient in both  $\alpha$ v $\beta$ 6 and  $\alpha$ v $\beta$ 8, or lacking the integrin binding site in the latency-associated peptide (LAP), develop phenotypes closely resembling TGF- $\beta$  knockouts<sup>12, 13</sup>. We have previously reported that mice lacking  $\alpha$ v integrins in myeloid cells have reduced numbers of intestinal Tregs and develop spontaneous colitis, and that DCs from the MLN of  $\alpha$ v-deficient mice are impaired in their ability to induce Tregs in culture<sup>14</sup>. To determine whether this was due to specific defects in CD103<sup>+</sup> DCs, CD103<sup>+</sup> and CD103<sup>-</sup> DCs were sorted from the MLN of  $\alpha$ v-tie2 and control mice and cultured with naïve FoxP3-GFP T cells. CD103<sup>+</sup> DCs from  $\alpha$ v-knockout mice did not exhibit the enhanced generation of Tregs seen in CD103<sup>+</sup> DCs from control mice, and instead induced similar numbers of Tregs to CD103<sup>-</sup> DCs (Figure 2A–B). We then tested the ability of  $\alpha$ v-deficient DCs to activate TGF- $\beta$ . In the presence of latent TGF- $\beta$ ,  $\alpha$ v-deficient CD103<sup>+</sup> DCs did not generate as many Tregs as CD103<sup>+</sup> DCs from wild-type mice, and only produced similar proportions to CD103<sup>-</sup> DCs, as we had seen in cultures with serum (Figure 2C). In contrast, active TGF- $\beta$  stimulated Treg generation by  $\alpha$ v-deficient CD103<sup>+</sup> DCs to levels close to those seen in control CD103<sup>+</sup> DCs (Figure 2D). The small difference in Treg generation between CD103<sup>+</sup> DCs from control and  $\alpha$ v-deficient cells in the presence of active TGF- $\beta$  could be rescued by addition of RA (Figure 2E) suggesting that  $\alpha$ v-deficient DCs may have a slightly reduced ability to generate RA.

However, the ability of  $\alpha\text{v}$ -deficient CD103<sup>+</sup> DCs to generate Tregs in the presence of latent TGF- $\beta$  was not rescued by the addition of RA (Figure 2E). These data therefore indicate that MLN CD103<sup>+</sup> DCs require  $\alpha\text{v}$  integrins to activate latent TGF- $\beta$  and generate Tregs. We interpret the small induction of FoxP3<sup>+</sup> Tregs by CD103<sup>-</sup> DCs or  $\alpha\text{v}$ -deficient cells in cultures with latent TGF- $\beta$  as a result of a small amount of contaminating active TGF- $\beta$  in the latent TGF- $\beta$  preparation, although it is possible that this results from activation of latent TGF- $\beta$  by an  $\alpha\text{v}$ -independent mechanism.

As MLN CD103<sup>+</sup> DCs originate in the intestinal lamina propria, we tested whether all intestinal DCs exhibited a similar ability to activate TGF- $\beta$ , or whether this was similarly restricted to CD103<sup>+</sup> DCs. Consistent with a specialized function for CD103<sup>+</sup> DCs in both intestine and MLN, CD103<sup>+</sup> DCs from both small intestine and colon induced more Tregs than CD103<sup>-</sup> DCs in the presence of latent TGF- $\beta$  (Figure 3). Furthermore, as for MLN DCs, this was dependent on expression of  $\alpha\text{v}$  integrins, and  $\alpha\text{v}$ -deficient DCs from the intestine induced similar numbers of FoxP3<sup>+</sup> Tregs to CD103<sup>-</sup> DCs from either  $\alpha\text{v}$ -knockout or control mice (Figure 3). Together these data show that the preferential ability of intestinal CD103<sup>+</sup> DCs to promote Treg generation *in vitro* is dependent on expression of  $\alpha\text{v}$  integrins and appears to be due to an increased activation of latent TGF- $\beta$ .

### **$\alpha\text{v}$ is required for intestinal conversion of peripheral T cells to FoxP3<sup>+</sup> Tregs**

CD103<sup>+</sup> DCs are thought to be responsible for the generation of gut-homing Tregs from naïve T cells *in vivo*.<sup>10, 11</sup>  $\alpha\text{v}$ -conditional knockout mice have fewer intestinal FoxP3<sup>+</sup> Tregs than control littermates, and develop spontaneous colitis<sup>14</sup>. We have hypothesized that this is due to failure of peripheral conversion of CD4<sup>+</sup> T cells specific for intestinal antigens to FoxP3<sup>+</sup> Tregs (so-called induced Tregs [iTregs]), which are required to regulate intestinal immunity. In contrast, Tregs in lymphoid organs, which probably represent primarily 'natural Tregs' of thymic origin are unaffected by  $\alpha\text{v}$  deficiency<sup>14</sup>. Consistent with this hypothesis, we recently showed that naïve CD4<sup>+</sup> T cells adoptively transferred into  $\alpha\text{v}$ -deficient lymphopenic SCID recipients do not differentiate into intestinal FoxP3<sup>+</sup> Tregs, unlike T cells transferred into  $\alpha\text{v}$ -expressing SCID mice<sup>15</sup>.

To test directly for the requirement for  $\alpha\text{v}$  integrins in iTreg generation, we used a model of oral antigen-dependent Treg conversion<sup>8, 10, 11</sup>.  $\alpha\text{v}$ -tie2<sup>14</sup> and littermate control mice were adoptively transferred with T cells from DO11.10 mice that express a transgenic TCR specific for ovalbumin. Recipients were then fed ovalbumin dissolved in drinking water for five consecutive days. After oral ovalbumin administration, DO11.10<sup>+</sup> T cells were readily detected in small intestine and colon lamina propria, as well as the MLN and PP, of control recipients. A significant proportion of these cells expressed FoxP3 (between 10 and 40% depending on tissue examined; Figure 4 and data not shown), representing Tregs that had converted from naïve T cells in the intestinal environment in response to oral antigen<sup>8, 10, 11</sup>. In contrast, very few converted FoxP3<sup>+</sup> DO11.10 cells were found in the intestine and intestinal lymph organs of  $\alpha\text{v}$ -tie2 recipients (less than 8% in all tissues; Figure 4). As reported in other studies, almost no FoxP3<sup>+</sup> DO11.10 cells were recovered from the intestine or lymph nodes of mice that did not receive ovalbumin, regardless of their genotype (data not shown). Thus, the induction of FoxP3<sup>+</sup> Tregs in response to oral antigen was substantially impaired in  $\alpha\text{v}$ -knockout mice.

### **$\alpha\text{v}\beta 8$ integrin is preferentially expressed on DCs from mucosal tissues**

The data presented above suggested that the preferential ability of MLN CD103<sup>+</sup> DCs to generate Tregs *in vitro* and *in vivo* was due to their ability to activate TGF- $\beta$  via  $\alpha\text{v}$  integrins.  $\alpha\text{v}$  associates with 5 different  $\beta$  subunits,  $\beta 1$ ,  $\beta 3$ ,  $\beta 5$ ,  $\beta 6$  and  $\beta 8$ , and all have been shown to bind and activate TGF- $\beta$ , albeit through different mechanisms and in different cell



types<sup>16</sup>. To identify the specific  $\alpha$ v integrin responsible for the preferential activation of TGF- $\beta$ , we analyzed the expression of  $\alpha$ v and pairing  $\beta$  integrins in DCs. We first compared integrin expression by MLN DCs with DCs from the spleen, by RT-PCR.  $\alpha$ v,  $\beta$ 3 and  $\beta$ 5 integrins (encoded by genes *Itgav*, *Itgb3* and *Itgb5* respectively) were all expressed at equivalent levels in spleen and MLN DCs and  $\beta$ 6 could not be detected in any DCs (Figure 5A). Strikingly, expression of  $\beta$ 8 (*Itgb8*) was only detected in MLN DCs but not in spleen DCs. This was confirmed using QRT-PCR, which is significantly more sensitive than rt-PCR (Figure 5C). To determine whether these differences in gene expression led to changes in protein, we measured expression of  $\beta$ 8 protein by western blot (suitable antibodies against mouse  $\alpha$ v $\beta$ 8 are not available for FACs).  $\beta$ 8 could be readily detected in DCs from the MLNs but not spleen (Figure 5D) confirming that changes in  $\beta$ 8 gene expression correlate with  $\beta$ 8 protein levels.

We then tested whether  $\beta$ 8 was differentially expressed between MLN DC subpopulations. CD11c<sup>+</sup> DCs from the MLNs were sorted by FACs based on CD103 and CD8 $\alpha$  expression, and  $\beta$ 8 expression measured (Figure 5A–C). The vast majority of  $\beta$ 8 expression seen in MLN DCs was from the CD103<sup>+</sup> subset and little or no expression could be seen by RT-PCR in CD8 $\alpha$ <sup>+</sup> or CD8 $\alpha$ /CD103 double negative cells (the majority of which correspond to CD11b<sup>+</sup> DCs). This was confirmed by QRT-PCR analysis, which indicated at least 10-fold higher levels  $\beta$ 8 in CD103<sup>+</sup> DCs compared with other MLN DCs.

Finally, we analysed  $\alpha$ v and  $\beta$  integrin expression in DC subsets from peripheral lymph nodes, such as those that drain the skin, to determine whether  $\beta$ 8 expression was limited solely to DCs derived from the intestine. We found that  $\beta$ 8 was also selectively expressed by the CD103<sup>+</sup> subpopulation of DCs from peripheral lymph nodes (Figure 5E), indicating a potential common role in generating Treg cells for CD103<sup>+</sup> DCs in peripheral lymph nodes. However, it should be noted that expression was considerably lower than seen in MLN CD103<sup>+</sup> DCs. Indeed, it is interesting that DCs could be readily clustered into the CD103<sup>+</sup>, CD8 $\alpha$ <sup>+</sup> and DN subsets based solely on relative expression of  $\alpha$ v and pairing  $\beta$  integrins, the only exception being CD103<sup>+</sup> DCs from the spleen.  $\beta$ 8 could not be detected by QRT-PCR of RNA in this small population of cells and they instead clustered with CD8 $\alpha$ <sup>+</sup> DCs in our analysis.

Analysis of expression of  $\alpha$ v integrins therefore indicated that the preferential ability of CD103<sup>+</sup> DCs to activate TGF- $\beta$  and generate Tregs is most likely due to expression of  $\alpha$ v $\beta$ 8, which is present at much higher levels on this DC subset in the intestinal lymph nodes. This is in agreement with studies of  $\beta$ 8 integrin conditional knockout mice, which have a similar phenotype to  $\alpha$ v knockouts, developing colitis associated with loss of intestinal Tregs. Hence  $\beta$ 8 appears to be the critical partner for  $\alpha$ v in activation of TGF- $\beta$  by DCs, conferring the preferential ability of CD103<sup>+</sup> DCs to generate Tregs in the presence of latent TGF- $\beta$ .

## Discussion

A specific population of intestinal DCs, marked by expression of CD103, are emerging as important regulators of intestinal immunity through their ability to promote conversion of naïve T cells to FoxP3<sup>+</sup> regulatory T cells that home to the intestine. Here we present data showing that the increased ability of CD103<sup>+</sup> DCs to generate Tregs both *in vitro* and *in vivo* is completely dependent on the expression of  $\alpha$ v integrins. This is due to an increased ability of CD103<sup>+</sup> DCs to activate latent TGF- $\beta$ , and CD103<sup>+</sup> DCs are uniquely specialized for this role through expression of high levels of  $\alpha$ v $\beta$ 8, which is restricted to this lineage of cells.

These data build on our previous work<sup>14</sup> in which we demonstrated that deletion of  $\alpha$ v from immune cells caused spontaneous colitis in mice. This was associated with a reduction in the

numbers of FoxP3<sup>+</sup> Tregs in the intestine, but not in lymph nodes or spleen, which we postulated was due to the selective loss of iTregs<sup>14, 17</sup>. Critically, this was caused by the deletion of  $\alpha$ v, or the pairing  $\beta$ 8 subunit<sup>18</sup>, from DCs, suggesting that  $\alpha$ v $\beta$ 8 was essential for DCs to generate intestinal iTregs. Here we demonstrate that this is the case and identify CD103<sup>+</sup> DCs as the  $\alpha$ v $\beta$ 8 expressing DCs responsible. We therefore propose a model in which activation of latent TGF- $\beta$  is tightly controlled by DCs, which 'license' TGF- $\beta$  signaling to interacting naïve T cells in an antigen-dependent manner. This is supported by our recent work showing that T cell responses to latent TGF- $\beta$  requires cognate interaction with  $\alpha$ v-expressing DCs<sup>15</sup>. This mechanism appears to be of particular importance in tissues such as the intestine which are rich in latent TGF- $\beta$ , and allows DCs to regulate TGF- $\beta$  signaling to DCs regardless of the source of TGF- $\beta$ . We have not directly addressed the source of TGF- $\beta$  in this study, relying on exogenous addition of latent or active forms. However, it is likely T cells represent a major source *in vivo* as deletion of *Tgfb1* in T cells alone led to a phenotype similar to that seen in DC-specific  $\alpha$ v and  $\beta$ 8 knockouts<sup>19</sup>.

We have recently reported that a similar  $\alpha$ v-mediated mechanism regulates the differentiation of Th17 cells, which share a common precursor and a requirement for TGF- $\beta$  early in development<sup>15</sup>. In that study, we did not identify the population of DCs required for TGF- $\beta$  activation in Th17 responses, although they almost certainly express  $\beta$ 8 as similar defects in Th17 cell generation were reported for DC-specific  $\beta$ 8 knockout mice<sup>20</sup>. Supportive of this, DCs associated with Th17 cell induction in response to commensal bacteria have also been shown to upregulate *itgav* and *itgb8* expression<sup>21</sup>. Upregulation of  $\beta$ 8 in DCs is therefore a prerequisite for generation of TGF- $\beta$  dependent T cell responses. It was striking to us that  $\beta$ 8 expression was highly restricted to intestinal CD103<sup>+</sup> DCs but was not seen in CD103<sup>+</sup> DCs from the spleen, suggesting that  $\beta$ 8 expression may be stimulated in response to signals encountered in the intestinal microenvironment. These may be derived from intestinal epithelial cells, which promote the ability of DCs to generate Tregs<sup>22</sup>, or from commensal bacteria<sup>21</sup>. Similar signals in skin, lung and other mucosal sites may also promote the expression of  $\beta$ 8 (Figure 5), and our observation that  $\alpha$ v-tie2 mice develop inflammation in multiple tissues, including the lung and respiratory tract, show that similar regulatory mechanisms are likely to exist throughout the body<sup>14</sup>.

RA has also been implicated in the preferential generation of intestinal iTregs by CD103<sup>+</sup> DCs<sup>10, 11</sup>. In addition to stimulating expression of gut homing receptors  $\alpha$ 4 $\beta$ 7 integrin and CCR6<sup>23, 24</sup>, RA enhances Treg generation through promotion and stabilization of FoxP3 expression. Our data indicate that  $\alpha$ v $\beta$ 8 and RA have complementary roles in Treg generation. Hence, DC  $\alpha$ v integrins are essential for generation of both iTreg and Th17 cells<sup>14, 15</sup> by mediating activation of TGF- $\beta$  whereas RA acts downstream of  $\alpha$ v to promote TGF- $\beta$ -driven Treg generation by stabilization of FoxP3 expression and reciprocal inhibition of Th17 differentiation<sup>25, 26</sup>. RA may therefore play a more important role in maintaining the appropriate balance between Treg and Th17 cells, than in initiating regulatory T cell responses.

Taken together our data support a model in which intestinal CD103<sup>+</sup> DCs are specialized for their role in inducing immune tolerance to oral antigens and the commensal microbiota through expression of  $\alpha$ v $\beta$ 8 integrin. These cells are uniquely placed to utilize the high levels of latent TGF- $\beta$  present in the intestine to promote immunoregulatory T cell responses in interacting T cells. Hence, Treg induction is restricted by availability of TGF- $\beta$ , by the lineage of the DC encountered and by the antigen specificity of the T cell. Our findings highlight the critical role of CD103<sup>+</sup> DCs in inducing and maintaining intestinal tolerance and identify modulation of  $\alpha$ v $\beta$ 8 expression or function as a potential therapeutic target in inflammatory bowel disease.

## Materials and Methods

### Mice

$\alpha\text{v-tie2}$  mice<sup>14, 15</sup> backcrossed to BALB/c or C57BL/6 backgrounds (10 generations) were used. FoxP3 eGFP reporter mice were obtained from Vijay Kuchroo (Brigham and Women's Hospital, Boston, MA)<sup>8</sup> and DO11.10 mice from Jackson Labs (Bar Harbor, ME). All animals were housed under specific pathogen free conditions at Massachusetts General Hospital. Animal experiments were performed under appropriate licenses within local and national guidelines for animal care.

### In vitro Treg generation assay

CD4<sup>+</sup> T cells were sorted by magnetic separation (CD4<sup>+</sup> pre-enrichment kit, Stem Cell Technologies) from splenocytes of FoxP3-GFP mice. Naïve CD4<sup>+</sup>CD62L<sup>hi</sup>FoxP3-GFP<sup>-</sup> T cells were further sorted by FACS. For MLN DCs, MLNs were digested in RPMI1640 containing DNaseI (20 mg/ml) and Liberase TL (0.33 mg/ml; both from Roche) followed by incubation in PBS/2% BSA/10 mM EDTA. Filtered cell suspensions were enriched for DCs using an Optiprep gradient (SIGMA) and CD11c<sup>+</sup> CD103<sup>+</sup> and CD103<sup>-</sup> DCs further sorted by FACS. To prepare intestinal DCs, mice were first injected subcutaneously with B16 cells secreting Flt3-L<sup>27</sup> to increase yield of DCs. Lamina propria cells were prepared as described previously<sup>15</sup> and DCs enriched and FACS sorted as for MLN DCs. Naïve spleen T cells and MLN DCs were co-cultured in either RPMI 1640 medium supplemented with 10 % fetal bovine serum and 10 mM HEPES, or in serum free X-VIVO 15 medium (LONZA). Both media also contained 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µM 2-β-mercaptoethanol. Cells were cultured in 500 µl volume containing 1–2 × 10<sup>4</sup> DCs and 2.5–5 × 10<sup>4</sup> naïve CD4<sup>+</sup> T cells (1:5 DC:T cell ratio), in the presence of 0.5 µg/ml anti-CD3 antibody (145-2C11; BD Biosciences). Additional cytokines used were recombinant active-TGF-β1 (0.5 ng/ml) and purified human latent TGF-β (2 ng/ml; both from R&D Systems). Where stated cells were cultured in presence of 40 µg/ml blocking anti-TGF-β1, 2, 3 antibody (1D11; R&D Systems) or all-trans retinoic acid (100 nM; SIGMA). Cells were cultured for 4–5 days before analysis of Treg generation assessed by GFP expression.

### In vivo Treg generation

CD4<sup>+</sup> T cells were sorted from the spleen and peripheral lymph nodes of DO11.10 Tg mice by depletion (Miltenyi Biotec) and adoptively transferred into BALB/c-background  $\alpha\text{v-tie2}$  mice or littermate controls. Each mouse received 10<sup>6</sup> cells. Recipients were maintained on either normal drinking water, or a 1.5 % solution of ovalbumin (grade V; Sigma-Aldrich) for 5 days. On day 6, lymphocytes were isolated from MLNs, Peyer's patches and intestinal lamina propria of recipients<sup>15</sup> and FoxP3 expression in transferred cells assessed by FACS.

### $\alpha\text{v}$ integrin expression analysis

DCs from spleen, MLN and intestine as described above. PLN and Peyer's patch DCs cells were prepared as for MLN. DCs were then sorted by FACS based on CD11c, CD103 and CD8α directly into TRIZOL LS (SIGMA) for RNA extraction. cDNA was synthesized using High Capacity Reverse Transcription kit (ABiosystem) and RT-PCR was performed with Taq DNA polymerase kit (TaKaRa) and analyzed on 1.4 % agarose gel. For real-time PCR analysis,  $\alpha\text{v}$  and pairing β integrins levels were quantified using Eppendorf real-time PCR system according to manufacturer's instructions in SYBR green master mix (Applied Biosystems). Expression was normalized to the housekeeping gene β-actin.



## Western blotting

DCs were sorted (>95%) from spleen and MLN leukocytes using CD11c microbeads (Miltenyi Biotech) and lysed in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 % NP-40, 0.5 % Sodium deoxycholate, 0.1 % SDS, 5 mM EDTA) containing Complete protease inhibitor cocktail (Roche). Lysates were separated by MOPS-12 % PAGE, transferred to PVDF membrane and immunoblotted with antibodies against  $\beta$ 8 integrin<sup>28</sup> and  $\beta$ -actin (AC-74; SIGMA).

## Antibodies

The following antibodies were used: anti-CD11c-APC (HL3), anti-CD103-PE (M290), anti-CD8 $\alpha$ -FITC (53-6.7), anti-CD4-APC (RM4-5), DO11.10 clonotypic antibody (KJ-1.26), anti-CD62L-PE (MEL-14; all from BD Bioscience), anti-CD11b-FITC (M1/70) and anti-FoxP3 (FJK-16S) (eBioscience),

## Acknowledgments

*Grant Support:* This work was supported by grants from the Crohn's and Colitis Foundation of America and the Hood Pediatric Research Foundation (to AL-H), The National Institutes of Health (grant RO1AI79198 to LMS), the UK Medical Research Council (grant G0802069 to JS), the National Institutes of Health (grant RO1AI079198 to LMS), a Wellcome Trust travelling fellowship (to SM) and the Philippe Foundation and the Bettencourt-Schueller foundation (young scientist award to HP).

We would like to thank Joseph McCarty (MD Anderson Cancer Center, Houston, TX) for the kind gift of antibodies to  $\beta$ 8 integrin. We would also like to thank J Rodrigo Mora and Scott Snapper (MGH) for helpful discussion and members of the Mora and Snapper laboratories for technical advice and reagents. We thank David Dombkowski (MGH) for FACs sorting.

## Abbreviations

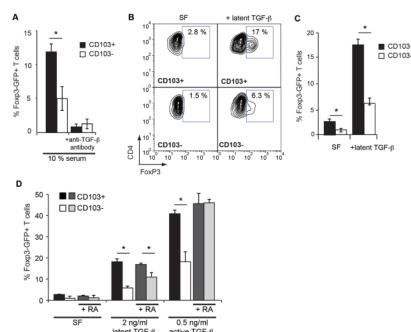
<b>DC</b>	Dendritic Cell
<b>FBS</b>	Fetal Bovine Serum
<b>iTreg</b>	induced regulatory T cell
<b>LAP</b>	Latency Associated Peptide
<b>MLN</b>	mesenteric lymph node
<b>RA</b>	Retinoic Acid
<b>Treg</b>	regulatory T cell
<b>TGF-<math>\beta</math></b>	transforming growth factor beta

## References

1. Sansonetti PJ, Medzhitov R. Learning tolerance while fighting ignorance. *Cell*. 2009; 138:416–20. [PubMed: 19665961]
2. Littman DR, Rudensky AY. Th17 and regulatory T cells in mediating and restraining inflammation. *Cell*. 2010; 140:845–58. [PubMed: 20303875]
3. Xavier RJ, Podolsky DK. Unravelling the pathogenesis of inflammatory bowel disease. *Nature*. 2007; 448:427–34. [PubMed: 17653185]
4. Powrie F, Read S, Mottet C, Uhlig H, Maloy K. Control of immune pathology by regulatory T cells. *Novartis Found Symp*. 2003; 252:92–8. discussion 98–105, 106–14. [PubMed: 14609214]
5. Bluestone JA, Abbas AK. Natural versus adaptive regulatory T cells. *Nat Rev Immunol*. 2003; 3:253–7. [PubMed: 12658273]

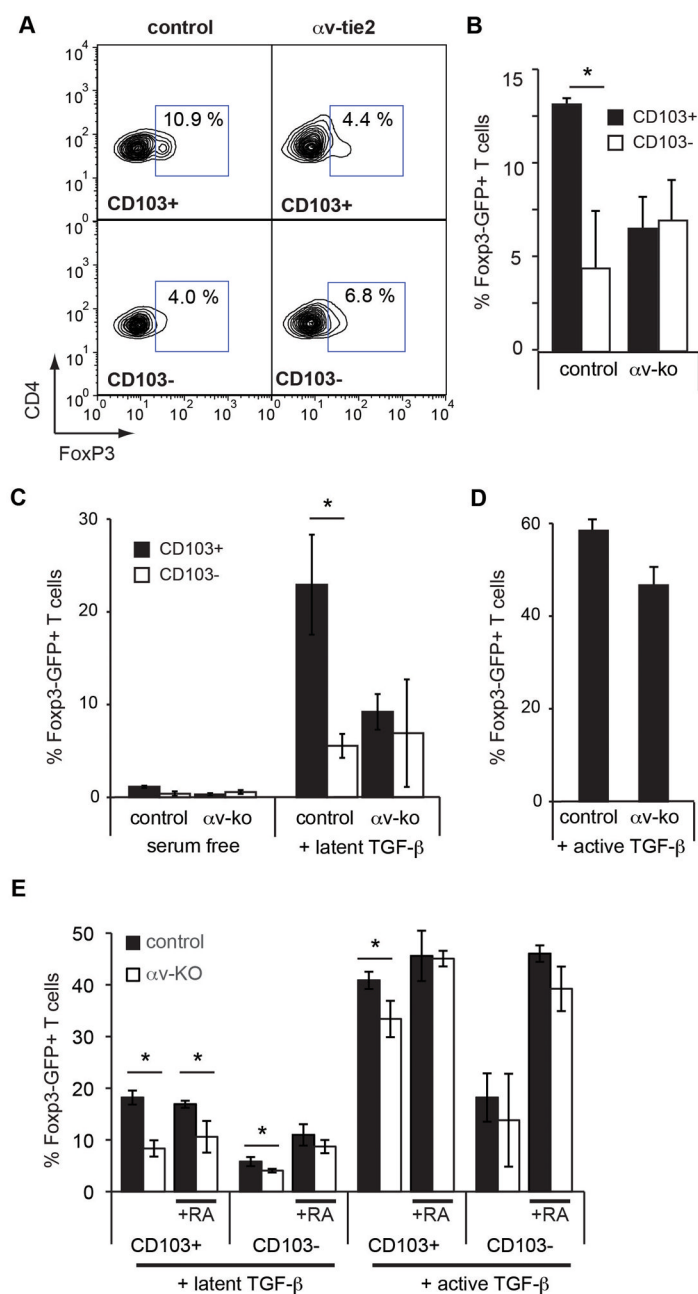
6. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. *Nat Immunol.* 2005; 6:1219–27. [PubMed: 16244650]
7. Knoechel B, Lohr J, Kahn E, Bluestone JA, Abbas AK. Sequential development of interleukin 2-dependent effector and regulatory T cells in response to endogenous systemic antigen. *J Exp Med.* 2005; 202:1375–86. [PubMed: 16287710]
8. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature.* 2006; 441:235–8. [PubMed: 16648838]
9. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med.* 2003; 198:1875–86. [PubMed: 14676299]
10. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *J Exp Med.* 2007; 204:1775–85. [PubMed: 17620362]
11. Coombes JL, Siddiqui KR, Arancibia-Carcamo CV, Hall J, Sun CM, Belkaid Y, Powrie F. A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. *J Exp Med.* 2007; 204:1757–64. [PubMed: 17620361]
12. Aluwihare P, Mu Z, Zhao Z, Yu D, Weinreb PH, Horan GS, Violette SM, Munger JS. Mice that lack activity of {alpha}v{beta}6- and {alpha}v{beta}8-integrins reproduce the abnormalities of Tgfb1- and Tgfb3-null mice. *J Cell Sci.* 2009; 122:227–32. [PubMed: 19118215]
13. Yang Z, Mu Z, Dabovic B, Jurukovski V, Yu D, Sung J, Xiong X, Munger JS. Absence of integrin-mediated TGF-beta 1 activation in vivo recapitulates the phenotype fo TGF-beta 1-null mice. *Journal of Cell Biology.* 2007; 176:787–793. [PubMed: 17353357]
14. Lacy-Hulbert A, Smith AM, Tissue H, Barry M, Crowley D, Bronson R, Roes J, Savill J, Hynes RO. Ulcerative Colitis and Autoimmunity Induced by Loss of Myeloid Alpha (v) Integrins. *Proc Natl Acad Sci U S A.* 2007; 104:15823–15828. [PubMed: 17895374]
15. Acharya M, Mukhopadhyay S, Paidassi H, Jamil T, Chow C, Kissler S, Stuart LM, Hynes RO, Lacy-Hulbert A.  $\alpha$ v integrin expression by DCs is required for Th17 cell differentiation and development of experimental autoimmune encephalomyelitis in mice. *J Clin Invest.* 2010; 120:4445–52. [PubMed: 21099114]
16. Wipff PJ, Hinz B. Integrins and the activation of latent transforming growth factor beta1 - An intimate relationship. *Eur J Cell Biol.* 2008
17. Paidassi H, Acharya M, Lacy-Hulbert A. Alpha(v) integrins license regulatory T cells to apoptotic cells and self antigens. *Annals New York Academy of Sciences.* 2010; 1209:68–76.
18. Travis MA, Reizis B, Melton AC, Masteller E, Tang Q, Proctor JM, Wang Y, Bernstein X, Huang X, Reichardt LF, Bluestone JA, Sheppard D. Loss of integrin alpha(v)beta(8) on dendritic cells causes autoimmunity and colitis in mice. *Nature.* 2007; 449:361–365. [PubMed: 17694047]
19. Li MO, Wan YY, Flavell RA. T Cell-Produced Transforming Growth Factor-beta1 Controls T Cell Tolerance and Regulates Th1- and Th17-Cell Differentiation. *Immunity.* 2007; 26:579–91. [PubMed: 17481928]
20. Melton AC, Bailey-Bucktrout SL, Travis MA, Fife BT, Bluestone JA, Sheppard D. Expression of alphavbeta8 integrin on dendritic cells regulates Th17 cell development and experimental autoimmune encephalomyelitis in mice. *J Clin Invest.* 2010; 120:4436–44. [PubMed: 21099117]
21. Atarashi K, Nishimura J, Shima T, Umesaki Y, Yamamoto M, Onoue M, Yagita H, Ishii N, Evans R, Honda K, Takeda K. ATP drives lamina propria T(H)17 cell differentiation. *Nature.* 2008; 455:808–12. [PubMed: 18716618]
22. Iliiev ID, Mileti E, Matteoli G, Chieppa M, Rescigno M. Intestinal epithelial cells promote colitis-protective regulatory T-cell differentiation through dendritic cell conditioning. *Mucosal Immunol.* 2009; 2:340–50. [PubMed: 19387433]
23. Mora JR, Bono MR, Manjunath N, Weninger W, Cavanagh LL, Roseblatt M, Von Andrian UH. Selective imprinting of gut-homing T cells by Peyer's patch dendritic cells. *Nature.* 2003; 424:88–93. [PubMed: 12840763]

24. Iwata M, Hirakiyama A, Eshima Y, Kagechika H, Kato C, Song SY. Retinoic acid imprints gut-homing specificity on T cells. *Immunity*. 2004; 21:527–38. [PubMed: 15485630]
25. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, Cheroutre H. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*. 2007; 317:256–60. [PubMed: 17569825]
26. Xiao S, Jin H, Korn T, Liu SM, Oukka M, Lim B, Kuchroo VK. Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J Immunol*. 2008; 181:2277–84. [PubMed: 18684916]
27. Mora JR, Iwata M, Eksteen B, Song SY, Junt T, Senman B, Otipoby KL, Yokota A, Takeuchi H, Ricciardi-Castagnoli P, Rajewsky K, Adams DH, von Andrian UH. Generation of gut-homing IgA-secreting B cells by intestinal dendritic cells. *Science*. 2006; 314:1157–60. [PubMed: 17110582]
28. McCarty JH, Lacy-Hulbert A, Charest A, Bronson RT, Crowley D, Housman D, Savill J, Roes J, Hynes RO. Selective ablation of {alpha}v integrins in the central nervous system leads to cerebral hemorrhage, seizures, axonal degeneration and premature death. *Development*. 2005; 132:165–176. [PubMed: 15576410]



### Figure 1. CD103<sup>+</sup> DCs promote Treg generation in the presence of latent TGF-β

Naïve CD4<sup>+</sup> FoxP3<sup>-</sup> T cells were cultured with FACS-sorted CD103<sup>+</sup> and CD103<sup>-</sup> DCs from MLNs in the presence of anti-CD3. The proportion of FoxP3<sup>+</sup> T cells generated was measured after 5 days by FACS. (A) Cells were cultured in medium containing 10% fetal calf serum and anti-CD3, with or without the addition of neutralizing antibodies against TGF-β (TGF-β ab). (B–D) Cells were cultured in serum-free medium, with or without the addition of latent TGF-β, active TGF-β or RA as indicated. Representative FACS plots are shown in (B). Cells are gated on CD4<sup>+</sup> cells, FoxP3 gates are indicated (gate position was set to give 0% positive cells in unstained samples). (C,D) show data from all samples in the same experiment. In all cases data points show mean ± standard deviation of at least three separate DC: T cell cultures and similar results were seen in three (C) or two (D) independent experiments. \*,  $p < 0.05$ , student's t-test.

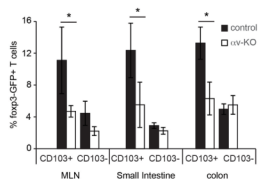


**Figure 2. Enhanced generation of Tregs and TGF- $\beta$  activation by MLN CD103<sup>+</sup> DCs is dependent on  $\alpha v$  integrins**

Naïve CD4<sup>+</sup> FoxP3<sup>+</sup> T cells were cultured with FACS-sorted CD103<sup>+</sup> and CD103<sup>-</sup> DCs from MLNs of  $\alpha v$ -tie2 ( $\alpha v$ -ko) and control mice in the presence of anti-CD3. The proportion of FoxP3<sup>+</sup> T cells generated was measured after 5 days by FACS. (**A**, **B**) Cells were cultured in medium containing 10% fetal calf serum and anti-CD3. Representative FACS plot are shown in (**A**), gated on CD4<sup>+</sup> cells, FoxP3 gates are indicated (gates position was set to give 0% positive cells in unstained samples) and data from one experiment are shown in (**B**). (**C**–**E**) Cells were cultured in serum-free medium, with or without the addition of latent TGF- $\beta$ , active TGF- $\beta$  and RA as indicated. In all cases data points show mean  $\pm$  standard deviation

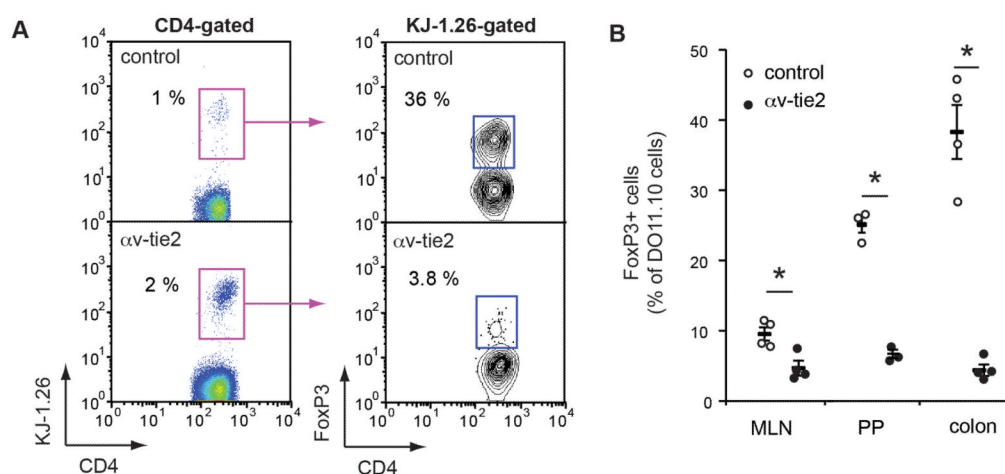


of at least three separate DC: T cell cultures and similar results were seen in three (**B–D**) or two (**E**) independent experiments. \*,  $p < 0.05$ , student's t-test.

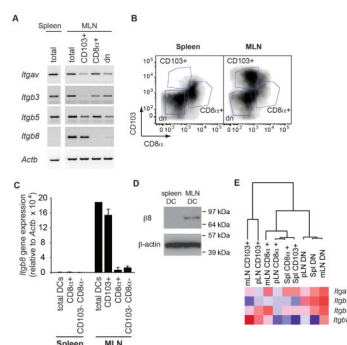


**Figure 3. Enhanced generation of Tregs and TGF- $\beta$  activation by intestinal CD103<sup>+</sup> DCs also requires  $\alpha$ v integrins**

Naïve CD4<sup>+</sup> FoxP3<sup>-</sup> T cells were cultured with FACS-sorted CD103<sup>+</sup> and CD103<sup>-</sup> DCs from MLNs, small intestine and colon lamina propria of Flt3-L expanded  $\alpha$ v-tie2 ( $\alpha$ v-ko) and control mice in the presence of anti-CD3 and latent TGF- $\beta$  in serum-free medium. The proportion of FoxP3<sup>+</sup> T cells generated was measured after 5 days by FACS. Figure shows mean  $\pm$  standard deviation of at least three separate DC:T cell cultures and similar results were seen in two independent experiments. \*,  $p < 0.05$ , student's t-test.



**Figure 4.  $\alpha$ v is required for conversion naïve T cells to FoxP3<sup>+</sup> Tregs in response to oral antigen** CD4<sup>+</sup> T cells from DO11.10 TCR transgenic mice were adoptively transferred to  $\alpha$ v-tie2 or littermate control mice. Recipients were fed ovalbumin in drinking water for 5 d. On day 6, conversion of transferred cells to FoxP3<sup>+</sup> Tregs was assessed in various tissues by intracellular staining for FoxP3 and FACS. **(A)** Isolated cells were gated on T cells by CD4 staining and size, and DO11.10-derived T cells identified by staining with the clonotypic antibody (KJ-1.26) (left panel). The proportion of DO11.10 T cells that had converted to Tregs was determined by FoxP3 staining (right panel). Representative staining of cells isolated from colonic lamina propria is shown. Numbers show percentage of DO11.10 and FoxP3<sup>+</sup> cells. **(B)** Percentage of FoxP3<sup>+</sup> cells within the transferred DO11.10 population in Peyer's patches (PP), mesenteric lymph nodes (MLN) and colonic lamina propria (colon). Circles represent individual mice ( $n=3$  mice/group for PP and colon,  $n=7$  for MLN), bars show mean  $\pm$  s.e.m. \*,  $p < 0.05$ , student's t-test. Data were combined from two independent experiments in which the same results were seen.



**Figure 5.  $\alpha$ v $\beta$ 8 is preferentially expressed by CD103<sup>+</sup> DCs from the mesenteric lymph nodes** DC subpopulations from various tissues were sorted by FACS and expression of  $\alpha$ v and pairing  $\beta$  subunits were measured by rt-PCR, QRT-PCR and western blot. (A–C) DCs from spleen and mesenteric lymph nodes were sorted on the basis of expression of CD11c, CD103 and CD8 $\alpha$ . (A) rt-PCR analysis of integrin and actin expression in total spleen and MLN DCs and subpopulations. (B) representative FACS plot of CD11c<sup>+</sup> DCs from spleen and MLN showing gating strategy for isolating indicated populations. (C) QRT-PCR analysis of the same samples in (B). Histogram shows mean  $\pm$  s.e.m. from three independent experiments. (D) Western blot of CD11c<sup>+</sup> DCs from spleen and MLN stained with an antibody against  $\beta$ 8. Also shown is staining for actin to confirm equal levels of protein loading. The same result was seen in two independent experiments. (E) Hierarchical clustering of DC subpopulations from spleen, MLN and peripheral lymph nodes (PLNs) based on integrin gene expression (determined by QRT-PCR analysis, using mean expression from at least two independent cell and RNA preparations per DC subset). DCs cluster into three principal groups discriminated by expression of  $\beta$ 8 and  $\beta$ 3 integrins.